Killer cell inhibitory receptors and

The leukocyte receptor gene complex

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I. Abbreviations

AML – acute myeloid leukemia
CLSF – C-type lectin superfamily
EBV – Epstein Bar virus
ERK – extracellular signal regulated kinase
EST – expressed sequence tag
GVHD – graft versus host disease
HLA – human leukocyte antigen
IgSF – immunoglobulin superfamily
ITAM – immunoreceptor tyrosine-based activation motif
ITIM – immunoreceptor tyrosine-based inhibition motif
LRC – leukocyte receptor gene complex
KIR – killer cell immunoglobulin-like receptor
LAIR – leukocyte-associated immunoglobulin-like receptor
LENG – LRC-encoded novel genes
LILR – leukocyte immunoglobulin-like receptor
MAPK – mitogen activated protein kinase
MHC – major histocompatibility complex
NK – natural killer
NKC – natural killer cell gene complex
ORF – open reading frame
PCR – polymerase chain reaction
PLC – phospholipase C
PVDF – polyvinyliden difluoride
RT – reverse transcriptase

SAP – slam associated adaptor protein
SDS – sodium dodecyl sulphate
SH2 – src homology 2 domain
SHP – src homology phosphatase
TCR – T cell receptor

II. Introduction

2.1. Natural killer cells and target recognition

In 1975, Kiessling et al. reported that spleen cells from adult, non-immunized mice displayed spontaneous lytic activity against certain tumor cell lines. The cytotoxic activity was excerted by a group of small undefined lymphocytes termed natural killer (NK) cells [1,2]. Natural killer (NK) cells are specialized in detecting aberrant cells in the body such as tumor transformed or virally infected cells, and the NK cells act as a first line of defense before the development of adaptive immunity. The NK cell induces lysis of the target cell in one of two ways: by generating cytokines, notably interferon γ, which activate other components of the immune system or by killing the target cell directly, a mechanism referred to as natural cytotoxicity. The function of the NK cell is not limited to being effector cells of the innate immune response, NK cells also act as regulatory cells and interact with dendritic cells in ways that seem to decide if and when adaptive immune responses should be made [3]. Great effort has been put forth to identify the receptors expressed by NK cells, the means by which the NK cells interact with their environment. Receptors are transmembrane anchored proteins, and during the past few years a plethora of new receptors expressed by NK cells have been identified. Structually,

most NK cell receptors are either members of the immunoglobulin superfamily (IgSF) or the C-type lectin superfamily (CLSF). The NK cell receptors can also be divided into two groups according to whether they transduce signals that inhibit or activate the NK cell. Primarily the receptors have been studied in the human and in rodents.

2.2. Killer cell inhibitory receptors (KIR)

In the mid 1980s experiments in mouse performed by Ljunggren and Kärre [4] and later in human [5-7], revealed that NK cells preferentially kill cells lacking the expression of MHC class I molecules, and based on these experiments the existence of inhibitory receptors regulating NK cell activation was predicted. The first killer cell immunoglobulin-like receptors (KIR) in human were identified in 1995 [8,9], and a surprising feature of the new family of receptors was the existence of both activating and inhibitory variants possibly recognizing the same ligands, HLA class I. Stimulation of the inhibitory receptors blocked the intracellular pathways that led to NK cell activation, and apparently a balance between stimulation and inhibition dictated the nature of the immune response [10]. NK cell tolerance of self was produced by the inhibitory signals overriding the activating signals when the cell interacted with a healthy autologus cell. It was also thought that NK cells killed target cells lacking MHC class I by default. However, this notion failed to explain why NK cells do not kill erythrocytes, which in humans do not express HLA class I. The recent identifications of a host of activating receptors such as NKp46, NKp30, NKp44 and the lectin-like NKG2D seem to resolve the problem of target recognition [11-14]. Other triggering surface receptors expressed by NK cells seem to rely on simultaneous co-engagement of another triggering receptor, and they appear to serve both activating and inhibitory functions depending on the availability of substrates for downstream signaling events. Two such receptors are 2B4 and NTB-A [15,16], they contain tyrosine based motifs in their cytoplasmic domains permitting association with SLAM-associated adaptor protein (SAP). Mutation in the *SAP* gene seen in human X-linked lymphoproliferative disease, results in life threatening infections with Epstein–Barr (EBV) and EBV-associated B-cell malignancies. In the absence of SAP both receptors convey inhibitory signals resulting in NK cell inactivation [15,17].

The KIR receptors recognize different different allelic groups of HLA-A, -B or -C molecules [8,18-20]. Importantly, each type of KIR receptor is expressed only by a subset of

NK cells. Each NK cell express at least one receptor specific for a self HLA class I molecule, and some data suggests that the coexpression of two or more receptors specific for HLA class I is rare [21,22]. This allows the whole NK cell pool to detect the loss of even a single HLA class I allelle on a cell, an event that frequently occurs on tumor transformed cells. However, *KIR2DL4* a KIR family member that shares structural features with both activating and inhibitory receptors, is uniquely expressed by all NK cell clones [23]. Contrary to the high specificity of the T-cell receptor, the KIR receptors proved to have a much broader specificity for MHC class I-alleles, e.g. a single receptor can recognize a majority of known allelic variants of HLA-C. Moreover, several observations indicate that the sequence of the peptide presented by the MHC class I molecule influences the binding of the different KIR members, and crystallographic structures show that the KIR receptor directly interacts with side chains belonging to both the MHC class I molecule and the peptide [24,25].

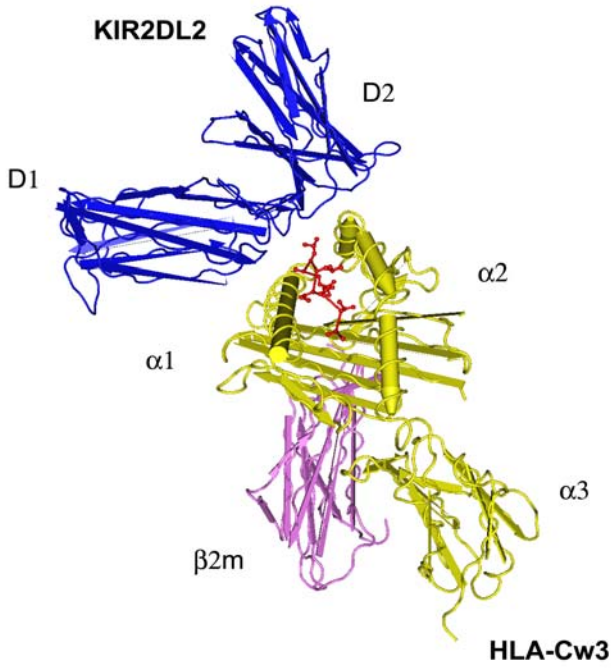


Figure 1:

Ribbon diagram of the KIR2DL2/HLA-Cw3 complex. The D1 and D2 Ig-domains of KIR2DL2 are colored blue, the α1, α2, and α3 domains of HLA-Cw3 are in yellow, the β2m molecule is violet and the presented peptide is colored red. (The diagram is constructed using Cn3D; *http://www.ncbi.nlm.nih.gov/Structure/CN3D/cn3d.shtml*, based on crystal structures; GenBank accession number 1EFX).

2.3. Intracellular signaling

The activating KIR receptors transmit signaling by binding to DAP12, a small transmembrane anchored adaptor protein that possesses an immunoreceptor tyrosine–based activation motif (ITAM) in the cytoplasmic domain. Phosphorylation of the ITAM facilitates the recruitment of tyrosine kinases Syk and Zap70. Downstream events include the phosphorylation of SLP-76, 3BP2, Shc, PI3-kinase, phospholipase C (PLC)-γ1 and PLC-γ2, the mobilization of Grb2, linker for the activation of T cells (LAT), Vav-1 and Vav-2, the elevation if intracellular Ca^{2+} levels, and the activation of Rho, Ras, p38, mitogen activated protein kinase (MAPK) and extracellular signal regulated kinase (ERK) [26-30].

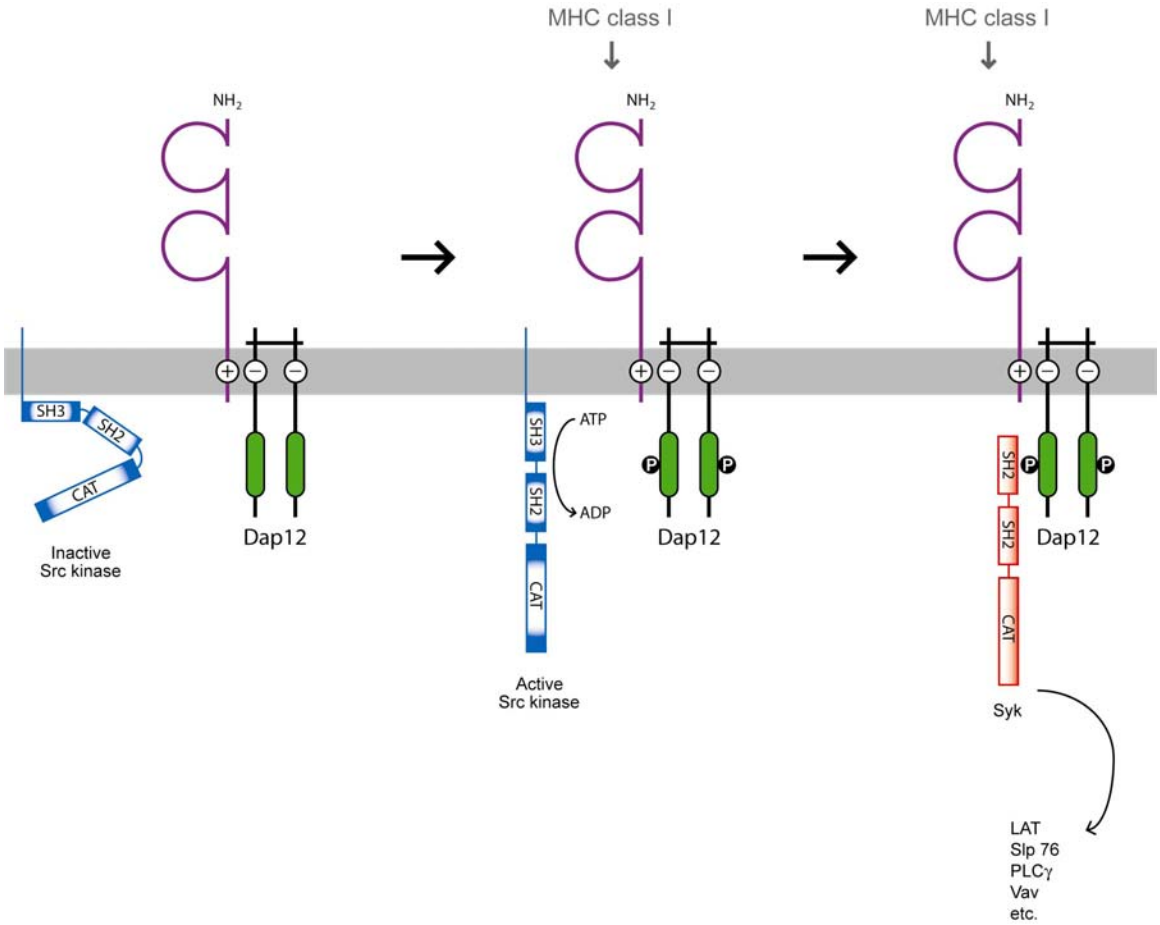


Figure 2:

A cartoon model of the activating KIR function. A prototypic 2 Ig-domain KIR is depicted, along with a Src family kinase and tyrosine substrates known to be in the DAP12 pathway. Cross-linking of activating KIRs leads to rapid and transient tyrosine phosphorylation of DAP12, although it is still unclear which tyrosine kinase is responsible. Upon phosphorylation, DAP12 acts as a recruitment point for the Syk/Zap70-family of kinases. The ITAM of DAP12 is colored green; SH2 represents Src homology 2 domain; CAT, catalytic domain.

In contrast, the inhibitory KIR receptors transduce intracellular signaling through a pair of immunoreceptor tyrosine-based inhibition motifs (ITIM) present in the cytoplasmic domain of the receptor. Ligation of the ITIM-bearing receptor results in the phosphorylation of the ITIMs and recruitment of the tyrosine phosphatases SHP-1 and SHP-2. The substrates of these phosphatases are still not identified, but they may depend upon the type of activating receptor being modulated [28]. However, SHP-1 possibly dephosphorylates ITAM polypeptides, Zap70, Syk, Vav-1 and other adaptor proteins responsible for the recruitment of downstream signaling molecules such as LAT and SLP-76.

2.4. Natural killer cell lectin-like receptors

In human the receptors that mediate NK cell inhibition were termed KIRs, but one failed to characterize KIR homologs in rodents. In contrast, rodents rely on the expanded Ly49 gene family to recognize MHC. The Ly49s are type II transmembrane glycoproteins, and are composed of an extracellular c-type lectin like domain connected to the transmembrane and intracellular domains by a stalk region [31]. The receptors exist exclusively as homodimers on the cell surface. Remarkably, even though KIRs and Ly49s are functional analogs they are structurally unrelated. Rodents and humans, it seems, developed different means to solve similar problems. The Ly49s recognize the MHC class I molecules H-2D and H-2K [32,33], and the repertoire of receptors expressed by an individual NK cell includes both activating and inhibitory Ly49s. The inhibitory Ly49s possess cytoplasmic ITIMs, and the activating variants contain a charged amino acid in the transmembrane domain, allowing association with the adaptor molecule DAP12 [34]. The genomes of non-rodent mammals, including humans, contain a single Ly49-like gene [35]. The *Ly49* variant in human is considered to be a pseudogene, although it appears to be functional in other species like orangutan, baboon and cow [36,37].

2.4.1. Ly49

The Ly49 gene family is clustered in the most telomeric region of the NK gene complex (NKC), localized on chromosome 6 in mice and on chromosome 4 in rats. However, different strains of mice differ significantly in the number of Ly49 genes as well as their sequences. The Ly49 cluster has been studied extensively in C57BL/6 mice, and the genomic sequence includes 15 complete Ly49 genes (*Ly49a-k*, *m*, *n*, *q* and *x*) and 4 gene fragments. Of these, 11 cDNA clones encoding functional receptors have been isolated [31,38]. The organization of the NKC has not been fully characterized in rats, but studies are well underway to reveal its structure (Nylenna et al, 2004).

The lack of KIR receptors in rodents was a bit of a mystery, and numerous attempts to identify orthologs were made. Finally, it was concluded that Ly49 genes predate the divergence of placental mammals, but expansion of the gene family was unique to the rodent lineage since they lacked KIR receptors. In non-rodent mammals however, KIR receptors replaced the function of the Ly49 gene and expanded in a similar manner. The rapid evolution of the genes probably reflected the changing selection by pathogens. Supposedly, the KIR receptor was lost from the genome of the mouse lineage sometime during the past 100 million years [39].

2.4.2. CD94/NKG2

A third family of MHC-specific receptors, CD94/NKG2, are lectin-like, disulfide-linked heterodimers expressed on both human and rodent NK cells. The *CD94* subunit is invariant, but NKG2 constitutes a family of at least four members; the inhibitory receptor *NKG2A*, and the activating receptors *NKG2C*, *NKG2D* and *NKG2E* [19]. CD94/NKG2A binds the non-classical class I molecule Qa-1^b in mice [40] and HLA-E in humans [41]. These non-classical molecules preferentially present leader peptides derived from classical MHC class I molecules, and thus are indicators of normal expression of MHC class I. NKG2D recognizes MICA and MICB in humans [42], MHC class I homologs that are upregulated on transformed or virally infected cells. Human NKG2D also binds ULBPs, a group of proteins that bind cytomegalovirus glycoprotein UL16 [43]. Rodent homologs of MICA and MICB have not been identified, but other molecules with weak homology to MHC class I including RAE-I and H-60 serve as ligands for mouse NKG2D [44].

2.5. The extended family of immunoglobulin-like receptors

The identification of numerous new receptors with structual and functional similarity to the KIR receptors have added to the complexity of NK cell interaction, but although numerous, signal transduction itself seems to converge on a few common biochemical pathways. Although several names have been assigned to these receptors, most have acquired official HUGO names. The majority of these new receptors in human belong to the leukocyte Ig-like receptor (LILR) family. Similar to the KIRs some of these receptors recognize MHC class I molecules.

2.5.1. Leukocyte immunoglobulin-like receptors

The LILR family members are glycoproteins with an extracellular ligand binding region composed of two, three or four Ig-like domains. The LILR family includes both inhibitory and activating members. The inhibitory variants have two or four ITIM motifs in their cytoplasmic region, and the activating receptors preferably associate with the FcεRIγ [45,46]. The LILR receptors may modulate both innate and adaptive immunity as they exhibit an exceptionally wide expression pattern. LILR variants are found on myeloid cells and granulocytes as well as subsets of T, B and NK cells [45]. Little is known about the genetic control of LILR transcription, although preliminary data suggests that some of the LILR variants show similar clonal expression pattern to KIRs [47]. LILRB1, LILRB2 and LILRA1 recognize HLA class I molecules. However, the LILR receptors are more promiscuous as they recognize a large number of HLA class I alleles, and in addition LILRB1 and LILRB2 also recognize nonclassical and viral orthologs of HLA class I [47-50]. The Gp49, NILR and PIR family of receptors are rodent genes most related to the human LILR family [51-54].

2.5.2. The human IgA receptor

The human IgA receptor, CD89, has two extracellular Ig-like domains, and a short cytoplasmic region devoid of any signal transduction domains. However, an arginine is present in the transmembrane region permitting association with the FcεRIγ [55]. FcεRIγ bears an ITAM motif in the cytoplasmic domain, allowing activatory functions. The receptor binds both IgA subclasses with low affinity, IgA1 and IgA2 differ by the absence of a thirteen amino acid sequence in the hinge region of the IgA2 molecule [56]. The receptor is expressed on neutrophils, eosinophils, monocytes/macrophages, dendritic cells and Kuppfer cells, and is defined a key role in mucosal defense.

2.5.3. Leukocyte-associated immunoglobulin-like receptors

The LAIR (leukocyte-associated immunoglobulin-like receptor) family constitutes together with *NKp46*, *CD89*, KIR and LILR the human LRC (leukocyte receptor gene complex) located on chromosome 19q13.4. *LAIR1* is a type I transmembrane protein with one Ig-like domain in the extracellular region, and the intracellular domain harbors two ITIM motifs. *LAIR2* is a putative secreted LAIR family member and shares 84% amino acid homology in the Ig- domain, but *LAIR2* lacks transmembrane and inctracellular domains, including ITIMs [57]. The LAIR family is expressed on a majority of peripheral blood mononuclear cells, including NK cells. Although, their role in regulating the immune response is unknown, the presence of ITIM motifs in *LAIR1* suggests the receptor suppresses cellular activatory functions.

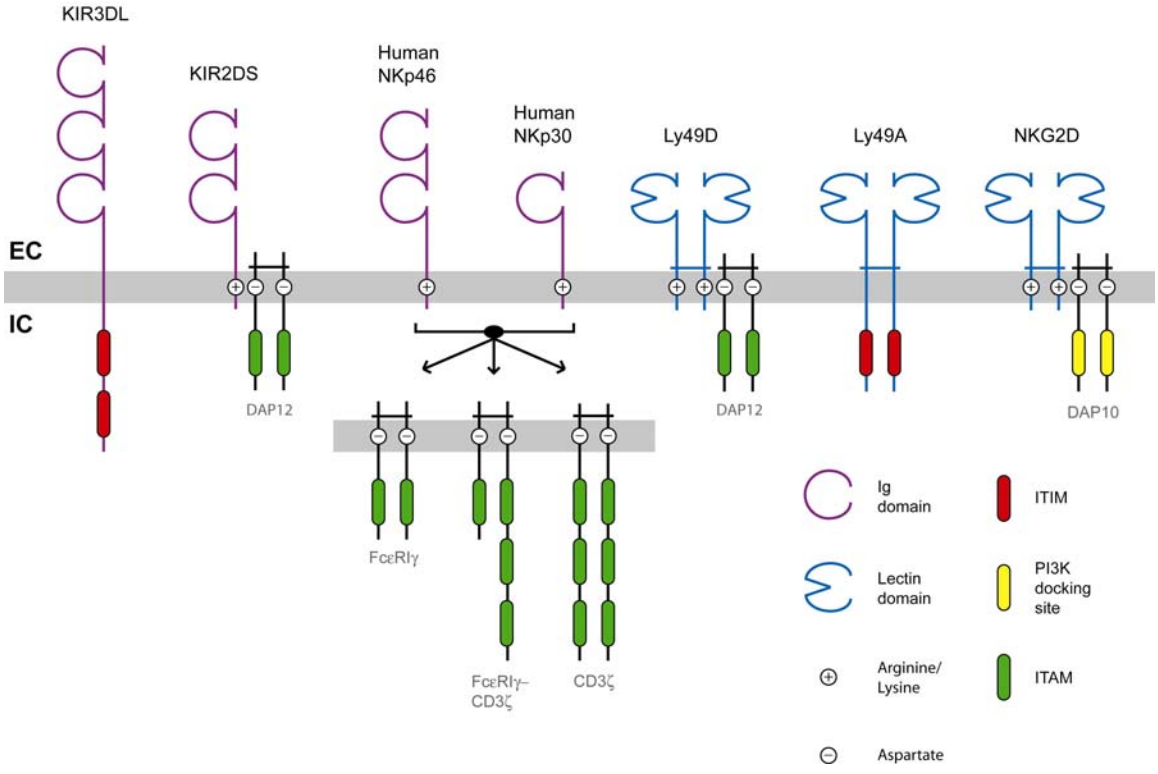


Figure 3:

Schematic structures of selected receptors expressed by NK cells. Human NKp46 and NKp30 can associate with homodimers or heterodimers of the FcεRIγ and CD3ζ adaptor proteins. EC, extracellular domains. IC, intracellular domains. The transparent shaded rectangle represents the transmembrane region. Horizontal interchain lines indicate disulphide bridges.

2.6. The immunological synapse

The NK immune synapse is the site of membrane apposition between the NK cell and target cell, and describes the clustering of receptors and their ligands. When NK cells encounter NK sensitive target cells, protein kinases such as Zap70, Syk and SLP-76 also localize to the intercellular interface together with the polarization of the microtubule organization center [58]. Some data indicates that in the presence of a ligand for an inhibitory KIR, SHP-1 enters the central zone of the synapse, however, when there is no ligand present, SHP-1 remains in the periphery [59]. Lipid rafts are microdomains in the cell membrane enriched in cholesterol and sphingolipids, and they have attracted considerable interest as they are thought to play an important role in many cellular processes including signal transduction [60]. Even though the organization of the immune synapse occurs independently of SHP-1, there are evidence suggesting that SHP-1 is critical for blocking lipid raft polarization and thereby NK cell cytotoxicity [61]. Thus, NK lipid raft polarization and NK immune synapse formation may reflect fundamentally different mechanisms. However, this is an issue of controversy, and additional experiments are needed to clarify the matter.

General techniques:

3.1. Polymerase chain reaction (PCR)

In PCR, DNA is immersed in a solution containing the DNA polymerase and unattached nucleotides (the subunits that DNA is composed of). Primers, short nucleotide sequences complementary to the 5' and 3' end of a desired DNA segment, are added to the solution. When the parental doubled stranded DNA is heated to 94°C the individual strands unwind and separate from each other. When the solution cools, the primers attach to the complementary sequences on the template DNA, and DNA polymerase will quickly synthesize new strands by adding free nucleotides to the primers resulting in the formation of two double stranded molecules. When this process is repeated, a strand that was formed with one primer binds to the other primer, resulting in a new strand restricted solely to the desired segment. Theoretically the number of strands will double for each time the process is repeated, this allows a researcher to amplify a segment, e.g. a gene, into a workable amount. Some common modification may improve the amplification and specifictity of the PCR. Hot start requires the polymerase to be added after denaturing of the DNA, thereby reducing false priming. Touchdown PCR effectively reduces unspecific primer hybridization by programming the first cycles to have an annealing temperature above the theoretical Tm for primer hybridization. The annealing temperature then gradually decreases for each cycle until Tm is reached. Specificity can

also be increased by performing a second PCR using primers located inside the first pair of primers, a technique referred to as nested PCR.

3.2. Gel electrophoresis

Gel electrophoresis takes advantage of the migration of charged molecules in solution in response to an electric field. Generally the samples are run in a support gel matrix such as agarose or polyacrylamide. These gels are porous and separate molecules by size by retarding the movement of larger molecules and allowing smaller molecules to migrate more freely. Because agarose gels are more rigid and easy to handle than polyacrylamide gels of the same concentration, agarose is used to separate large macromolecules such as nucleic acids. Polyacrylamide gels are more suitable to separate proteins that require a small gel pore size to retard migration. Proteins are amphoteric compounds and their net charge is determined by the pH of the medium in which they are suspended, in addition the net charge carried by a protein is independent of size. As a result the separation of proteins at a given pH and under non-denaturing conditions is determined both by the size and the charge of the molecules. Sodium dodecyl sulphate (SDS) is an anionic detergent that binds proteins in a mass ratio of 1.4:1 by wrapping around the polypeptide backbone. SDS denatures the protein and confers a negative charge to the polypeptide in proportion to its length thus allowing the separation of proteins by molecular weight. In contrast nucleic acids carry a fixed negative charge per unit length of molecule. Nucleic acids can be visualized by staining the gel with the fluorescent dye ethidium bromide (EtBr), EtBr binds tightly to DNA by intercalation. Protein gels can be stained with Comassie brilliant blue dyes, silver stain etc, or transferred to polyvinyliden difluoride (PVDF) or nitrocellulose membranes for detection with specific antibodies (Western blot).

3.3. Computer software

The genomic resources available are increasing at an astonishing pace. Genomic sequences from human, rat and mouse are available from the National Institute of Health (NIH) and through the EMBL project. The quality of the genomic assemblies is continuously improving, and the sequences can already be used to reveal a host of new genes. Searches can be based on peptide or amino acid sequence similarity using common algorithms such as BLAST or SSAHA, or analyzes of predicted genes generated

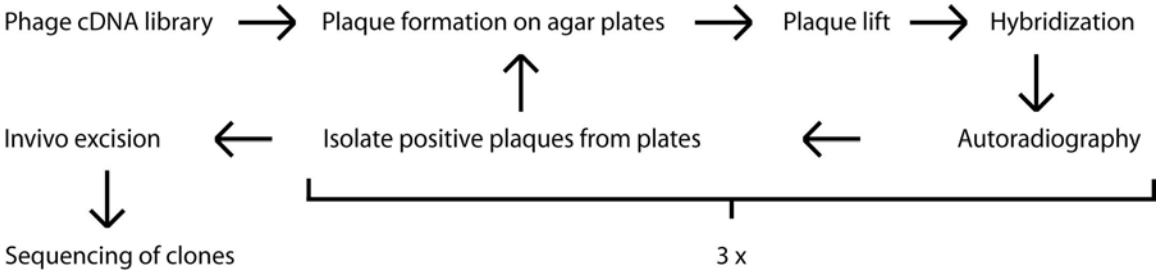
by automated genescans. A third option is to search the database of Expressed Sequence Tags (EST) for homology to already known genes. The ESTs are short single pass sequence reads from mRNA, and represent a snapshot of genes expressed in a given tissue. Novel genes can be characterized "in silico" using the GCG package accessible through the Norwegian National EMBnet node. Such sequences can serve as template for primer designs to obtain full length cDNA by PCR. Other powerful bioinformatic tools we commonly use at our laboratory are databases and software packages available through the Expert Protein Analysis System (ExPASy). Such programs can be used to predict the function of a gene and the structure of its domains. ClustalX, a multiple sequence alignment program, is useful to highlight conserved features and to generate phylograms.

3.4. Hybridization screening

Hybridization screening of a cDNA library has for a long time been a important tool, facilitating the identification of novel genes. mRNA is isolated from a given cell line or tissue, and converted to cDNA by reverse transcriptase (RT) PCR. The cDNA is then inserted into a plasmid or bacteriophage system. Most cDNA libraries used in our laboratory are based on the lambda ZAP vector system (Stratagene, La Jolla, CA). A mixture of bacteriophage and the bacterial strain MRF' is incubated for 15 minutes to let the phage attach to the cells, and the mixture is then plated on NZY plates. Plaques, sites of infected bacteria, are visible after overnight incubation, and the plaques are lifted onto nylon membranes (Millipore, Billerica, MA). The membranes are treated with NaOH to lyse bacteria and denature DNA, and then baked at 80°C so that DNA covalently attaches to the membrane. Hybridization with radioactive probes is the most sensitive method for detecting DNA bound to the membrane. The probe is usually a single strand cDNA containing the open reading frame (ORF) of a gene. Stringency determines the degree of sequence complementarity required for the probe to attach, and can be altered by adjusting the hybridization temperature and buffer composition. Prehybridization with sonicated salmon testes DNA prevents unspecific binding of the probe to unoccupied sites on the membrane. Usually hybridization is performed under low stringency conditions followed by high stringent washes to remove unspecifically bound probes. Sites of hybridization are visualized by autoradiography, and the corresponding plaque on the NZY plate is isolated. Phage extracted from the isolated agar is used in a secondary

screening, and finally a tertiary screening is performed as outlined above. The tertiary screening permits the isolation of bacteria solely infected with the phage containing the desired gene, and the lambda ZAP vector is subjected to in vivo excision. This releases the pBluescript phagemid contained in the lambda ZAP vector, and permits the insert to be characterized in a plasmid system.

Flow chart describing a bacteriophage cDNA library screening:



Hybridization protocol:

- Membranes are prehybridized for four hours in rotating bottles at 42°C in 50% deionized formamide, 5 x SSC, 50 mM NaP_i pH 6.5, 250 μg/ml sonicated salmon testis DNA (Sigma-Aldrich, St Louis, MO), 5 x Denhardt's solution and 0.1% SDS.
- The probe is radiolabeled with [^{32} P] dCTP using a random nonamer protocol (Megaprime DNA labeling system, Amersham International, Little Chalfront, GB) and hybridized in rotating bottles at 42°C for 16-20 hours in 50% deionized formamide, 5 x SSC, 50 mM NaP_i pH 6.5, 250 μg/ml sonicated salmon testis DNA (Sigma-Aldrich), 5 x Denhardt's solution and 0.1% SDS.

- Membranes are finally washed 4 x 5 min. at room temperature in 2 x SSC/0.1% SDS, followed by two high stringency washes at 50°C for 30 min. in 0.2 x SSC/ 0.1% SDS and then subjected to autoradiography.

3.5. Radiation hybrid (RH) mapping

Chromosomal localization of novel genes in the mouse and rat can easily be verified using a technique referred to as RH mapping. An RH panel clone is created by fusing an irradiated rat or mouse cell line with hamster cells, and the full panel consists of 106 RH clones, each clone containing unique combinations of fragments of rodent genomic DNA. The panel is analyzed by standard PCR technique using primers specific for the gene being investigated. Each gene will have their own exclusive fingerprint given by the 106 RH clones yielding a PCR product of desired length, indicating that the clone holds part of the genome containing the gene of interest. This fingerprint can then be compared to a framework of markers whose chromosomal localization are known. Our data are based on T31, a mouse/hamster RH panel (Research Genetics, Huntsville, AL), and the T55 rat/hamster panel (Research Genetics).

- The T31 mouse/hamster panel was analyzed with mouse *Kir3dl1* specific primers (5'- TGATGGGCCCTGTGCTGATGATG -3', 5'- CCTCGAATTTACCACTGTGGCTCCTG -3') for the presence of a 150-bp product, and with primers specific for mouse *NKp46* (5'- CCAAACCCAGCATCATGGTCACAAT -3', 5'- AGTTTCAGGGGGTTGCTCGACTT -3') yielding a 240-bp product.
- Chromosomal localization of rat *Kir3dl* was determined using gene specific primers (5'- TGCCTCGCCAAGTTATATTGTTCCTC -3', 5'- TCACTATGCGCTGAAATTTCATTAGGGT -3'), resulting in a 230-bp PCR fragment.

3.6. Southern Blot

Southern Blots can determine whether or not multiple related genes exist. A genomic southern blot is generated by digesting DNA with a given restriction enzyme, the DNA fragments are then separated by agarose gel electrophoresis and transferred to a nylon membrane. A single strand cDNA probe based on the gene of interest will hybridize with

complementary sequence, and can be visualized by labeling it with a radioactive isotope. A large number of bands in a lane indicate the existence of multiple related genes in the genome.

Protocol:

- DNA extracted from rat liver, digested with restriction endonuclease (New England Biolabs, Beverly MA) is subjected to horizontal agarose gel electrophoresis and blotted onto a Biotrans nylon membrane (ICN Biochemicals, Irvine, CA) as described elsewhere [62]. Hybridization to a radiolabeled probe is performed in 50% formamide, 5 x SSC, 50mM sodium phosphate pH 6.5, 250 μg/ml sonicated salmon testes DNA (Sigma-Aldrich), 5 x Denhardt's solution and 0.1% SDS at 42°C for 16-20 hours in a hybridization oven. Membranes are then washed 4 x 5 min. in 2 x SSC/0.1% SDS at room temperature, then 2 x 30 min. at 50°C in 0.1 x SSC/0.1% SDS. Where low stringency hybridization is required, hybridization temperature is lowered to 37°C, and final washes are carried out at 45°C in 1 x SSC/0.1% SDS for 2 x 30 min.

3.7. Reverse transcriptase (RT) PCR

RT-PCR is well suited to study the cellular expression of a certain gene and to quantitate the mRNA levels, i.e. establishing quality and quantity of gene expression. Total cellular RNA is isolated and subjected to first strand cDNA synthesis. The resultant cDNA mirrors the protein expression pattern of a certain cell type or tissue, and then functions as a template for regular PCR using primers specific for the gene of interest. Using primers from neighboring exons excludes the possibility of genomic contamination, as genomic DNA contains the intervening intron and yield fragments of greater length.

- First strand cDNA synthesis was performed by adding 1μg of total cellular RNA , 20U of RNasin ribonuclease inhibitor (Promega, Madison, WI), 0.75mM of each dNTP and 35ng of oligo$(dT)_{15}$ primer (Promega) to 20μl standard MMLV RT reaction buffer (Promega) and heating to 80°C for 5 min. Then, 200U of MMLV RT, RNase H minus (Promega) was added together with additional 16U RNasin, and the mixture was incubated at 37°C for 1 hour. The enzyme was inactivated at

81°C for 5 min., and PCR was performed on the cDNA with primers specific for the gene of interest.

- Mouse *Kir3dl1* expression was analyzed by PCR (35 cycles) using primers specific for the first (5'- CCTATCTGCCTGGCCAAGTTATG -3') and second (5'- TCACATTCCCTCCTGTATCCACC -3') Ig exons on first strand cDNA from C57BL/6 lymph node T cells (Pan T cell isolation kit; Miltenyi Biotec, Bergisch Gladbach, Germany), or IL-2 activated spleen NK cells cultured in rat IL-2 as described elsewhere [63].
- A primer pair specific for the first (5'- CTGTGCCTCGCCAAGTTATATTGTTC -3') and second (5'- AATGACACTCCAGGGTCACATTCTC -3') Ig exons of rat KIR3DL1 was used to analyze transcription by PCR (25 cycles), total RNA isolation was performed as described previously [64].

3.8. Generation of expression constructs

Epitope-tagged expression constructs are useful to dissect proximal signaling events. The Kozak sequence and ORF of a desired gene is amplified by PCR using primers containing restriction sites corresponding to the restriction sites incorporated into the multiple cloning site (MCS) of the expression vector. The PCR products are cloned into the pCR 2.1-TOPO vector (Invitrogen, San Diego, CA), and the fragment of interest released by digestion with restriction enzymes. The fragment can then be inserted into the MCS of a given expression vector.

<u>The mKIR3DL1 FLAG-tagged expression construct was generated as follows:</u>

- The ORF of mouse *Kir3dl1*, excluding leader sequence, was amplified with the primer pair; 5'- AAGCTTTTGCCAGCCATGCTGCTC -3', 5'- GGATCCGGTGCAGACATTCTTTGATCTC -3', containing HindIII and BamHI restriction sites respectively. The PCR products was cloned into the pCR2.1-TOPO vector (Invitrogen) according to manufacturers instructions, followed by digestion with HindIII and BamHI (New England Biolabs) to release the gene fragment with HindIII and BamHI overhangs. Finally the fragment were ligated into the expression vector pCMV-FLAG-1 (Sigma-Aldrich).

3.9. Transfection and immunoprecipitation

The transfection system often used in our laboratory is based on 293T cells, a human embryonic kidney cell line. Cells are transiently transfected using lipofectamine, a liposome formulation creating DNA-lipid complexes. Cationic lipids form such complexes due to the electric interactons between the negatively charged backbone of DNA and the positively charged lipids. These complexes fuse with the cell membrane by mechanisms not completely understood, and show a high transfection activity in most adherent cells. Cells are then lysed, the proteins are separated by electrophoresis in a polyacrylamide gel and then transferred to a nitrocellulose membrane. The membrane is incubated in a buffer containing a specific antibody against the protein of interest and visualized with a fluorescent dye labeling the antibody. To study protein-protein interactions the cell lysate can be subjected to immunoprecipitation. A desired protein and possible adaptor proteins can be precipitated by adding an antibody that specifically binds the particular protein. The antibody-protein complex is removed from solution by the addition of an insoluble form of an antigen binding protein such as protein G-sepharose beads. The beads are isolated by centrifugation, and after extensive washing immunoprecipitated material can be eluted from the beads by boiling it in a SDS-containing electrophoresis sample buffer. The eluate is then fractionated by SDS-PAGE and analyzed by Western blotting.

<u>Transfection protocol:</u>

- Adherent 293T cells are released in PBS containing 0.5mM EDTA, recultivated in RPMI 1640 (Invitrogen, San Diego, CA) with 1mM sodium pyruvate and then incubated for 24 hours. A 70-80% confluence upon transfection is considered optimal. Prior to transfection cells are washed three times in PBS, pH 7.4, in order to remove traces of serum.
- For transfections using 25 cm^2 flasks, 5 µg of plasmid DNA is added to 0.25 ml Opti-Mem (Invitrogen) and subsequently mixed with a solution of 0.25 ml Opti-Mem (Invitrogen) containing 20 µl Lipofectamin (Invitrogen). The mixture of the

two solutions is incubated at room temperature for 30 min., before being diluted in 1.8 ml Opti-Mem (Invitrogen) and finally added to the cells.

- After 6 hours incubation at 37°C, 2.5 ml of RPMI supplemented with 20% FCS is cautiously poured into the flask. Following another 18 hours, the cell medium is replaced with RPMI containing 10 % FCS. Facs flow analysis or cell lysis should be performed within the next 24-48 hours.

Western Blot protocol:

- Cells are lysed for 30 min. in 1% Igpal CA-630 (Sigma-Aldrich), 20mM TrisHCl, 100mM NaCl, 10mM NaF, 10mM $NaPP_i$, 1mM Na_3VO_4, 5mM EDTA and a protease inhibitor mixture (Sigma-Aldrich), followed by centrifugation; 13000 rpm in a microcentrifuge for 30 min at 4°C, and the supernatant (WCL) collected.
- The PVDF membrane (Millipore) is activated in methanol for 15-30 seconds, the gel and activated membrane are sandwiched between filter papers saturated with transfer buffer (25mM Tris base, 192mM Glycine, 10% methanol), and for each layer air bubbles are removed.
- Membranes are blocked with 5% non-fat dry milk in TBS containing 0.05% Tween20 for 1 hour (membranes intended for detection with 4G10, anti-phosphotyrosine mAb, are blocked with 3% Bovine Serum Albumin), followed by incubation with 1 μg/ml rabbit anti-FLAG ab (Sigma-Aldrich), or 0.2 μg/ml 4G10(Santa Cruz Biotechnology, Santa Cruz, CA) mAb fot at least 4 hours, succeeded by 6 x 5 min. washes in TBS. The membranes are then subjected to secondary incubation with goat anti-rabbit, or goat anti-mouse (anti igG2b) HRP conjugated mABs (Jackson ImmunoResearch Laboratories, West Grove, PA) for 1 hour and finally washed 6 x 5 min. in TBS. Chemiluminiscense detection is performed using SuperSignal substrate (Pierce, Rockford, IL) and captured on BioMax MR film (Eastman Kodak, Rochester, NY) or by digital camera, ImageStation 2000R (Eastman Kodak).

3.10. Molecular cloning of rodent LAIR, rodent LILR and rat CD89:

An unpublished cDNA sequence encoding mouse *Lair* was retrieved (GenBank accession number AF479685), and 5'UTR and 3'UTR gene specific primers were generated. Based

on sequence similarity to mouse *Lair*, exons encoding a rat *Lair* variant were recovered, including 5'UTR and 3'UTR sequences. Genes encoding rat and mouse *Lair* were amplified by standard touchdown PCR from spleen mRNA, harvested from PVG rats and C57BL/6 mice.

Mouse and rat *LilrA1* (leukocyte immunoglobulin-like receptor) were predicted on the basis of current mouse and rat genomic sequences (*http://www.ensembl.org/Mus_musculus/; http://www.ensembl.org/Rattus_norvegicus/),* exons were retrieved based on amino acid sequence similarity to human LILR sequences. 5'UTR and 3'UTR were obtained by extending the leader sequence upstream and the cytoplasmic sequence downstream respectively. The genes were amplified by PCR analogous to *Lair* as described above.

Rat *CD89* was retrieved by the detection of an EST containing the 3'UTR (GenBank accession number AI112904). A primer specific for 3'UTR was generated, and the cDNA amplified from Marathon-ready cDNA according to the manufacturer's instructions (BD Biosciences-Clontech, Franklin Lakes, NJ). The PCR product was sequenced and a 5'UTR primer generated. Finally, the gene was amplified from PVG spleen mRNA.

Rat *LilrA2* was detected following searches for genes with homology to rat *LilrA1*. Forward and reverse primers specific for the second Ig-like domain were generated (5'-TGGGTCGTAGAGTCGGTATGGTT -3'; 5'- AGAGTCCATACCAGTCAAGGGTC -3'), and the 3'- and 5'- gene fragments amplified from Marathon-Ready cDNA (BD Biosciences-Clontech). The partial genes were sequenced in order to disclose 3'UTR and 5'UTR, and the gene was then amplified from rat PVG mRNA as described above. No mouse equivalent to rat *LilrA2* has been detected.

The following 5'UTR and 3'UTR primers specific for *CD89*, *LilrA1*, *LilrA2* and *Lair* were generated:

Gene	**5'- UTR primer**	**3'- UTR primer**
Rat	GGTCTGAGGCCATATC	ATCATCAGGGGAAGCCAGGGTAG

CD89		
Rat *LilrA1*	TGCCATGACCTTCATCTTCACAGCC	GACCCAAACCTGACTGATGAAACAAGA
Rat *LilrA2*	TCCTGATGGTCACTGTAGTGTCATTT	CTATCCCAATTGCATAAGTGTGGAGT
Rat *Lair*	CAGACCTGGTAAGGTTGCTGGA	CTGGGCTCATTACACAACTGGG
Mouse *Lair*	TGCAGGGCTTCTGCTCTGAC	GAAGAGGCCTGGCAGTGAGA
Mouse *LilrA1*	TGCCATGACCTTCGTCTTCACAG	TGGCCATAGAAGAATTTTCCACCCAAG

Summary of paper

4.2. Cloning of KIR receptors in the mouse and rat:

By extensive searches in EST databases for homology to the human *KIR2DL4* gene, two promising candidates were retrieved in mouse (GenBank accession numbers: BB644731 and BB314335). This gave hope that the gene was actually expressed at some level. Mouse trace archives, repositories of raw sequence traces generated by the mouse sequencing project, was searched for homology to the EST sequence. Trace sequences were then assembled in a stepwise manner, and finally a genomic contig containing all the exons and introns of the KIR gene was completed. Based on these sequences PCR primers were synthesized, and the gene was successfully amplified by PCR from a mouse NK cell library. The gene shared many structural features with the *KIR3DL* variant in human, and consisted of three extracellular IgSF domains, a stem region, a transmembrane region lacking charged amino acids and a cytoplasmic region containing one pair of ITIM motifs.

This was prior to the first release of the mouse genome sequence and moreover, no genomic assemblies containing the gene were accessible at the time. We therefore

performed a hybridization screening using the full length PCR fragment as probe to identify closely related genes. Analysis of genomic trace sequences led to the suspicion that there existed at least two variants of the gene, but the hybridization screening only identified the variant obtained by PCR.

Parallel to the hybridization screening in mouse, the full length PCR clone was used as template for homology searches in rat trace archives. A similar cloning approach as described above was performed in the rat, and the hybridization screening identified several splice variants. Four different polyadenylation sites were observed, and cryptic splice sites were present in the second signal peptide exon, the transmembrane exon and importantly in the cytoplasmic exon leading to the loss of both ITIMs. In addition the transmembrane domain contained a trinucleotide repeat yielding an abnormally long transmembrane region. Altogether, these findings suggest that the gene may be a pseudogene, even though the large number of cDNA clones detected in the library screening and the readily detectable levels of mRNA demonstrated by RT-PCR indicate a high level of transcription in NK cells.

When the preliminary mouse genome sequence was published, it became clear that there exist two KIR variants in the mouse. We made several unsuccessful attempts to amplify the second gene by PCR from different NK cell libraries as well as RNA isolated from spleen and blood. The original ESTs were based on mRNA harvested from brain tissue, and the unexpected tissue expression might explain why cloning attempts have been futile. Analysis of rat genomic assemblies leaves no evidence in support for the existence of more than one KIR variant in rat.

4.2.1. Cellular expression:

Semiquantitative RT-PCR analysis demonstrated that *Kir3dl1* is selectively transcribed by NK cells in both mouse and rat. After additional cycles of PCR a weak band was detected in mouse lymph node T cells, possibly due to a small number of NK cells in the lymph node T cell preparation. No transcription was detected in rat T cells.

4.2.2. Chromosomal localization:

In the human the KIR family is located in the leukocyte receptor gene complex (LRC) on chromosome 19q13.4. The gene family comprises 16 members with several allelic variants and two pseudogenes. The KIR genes are clustered tightly in a head to tail fashion and span 100-150kb of genomic sequence. In the rat, RH screening localized the *Kir3dl1* receptor to the LRC on chromosome 1 as expected. Interestingly, screening a mouse/hamster RH panel reproducibly localized the mouse gene to the X chromosome. To exclude the possibility of a local error in the RH marker framework, the panel was screened with primers specific for mouse *NKp46*. *NKp46* localizes to the LRC in both human and mouse, and is closely linked to the KIR gene cluster in the human genome. The *NKp46* RH distribution pattern differed from the pattern obtained for *Kir3dl1*, and localized *NKp46* to the mouse LRC on chromosome 7. There is growing evidence for the translocation of a region in the mouse LRC on chromosome 7 that can explain these and other findings as will be discussed later.

4.2.3. Surface expression of KIR:

A mouse KIR3DL1-FLAG construct induced surface expression of KIR3DL1 when transiently transfected in 293T cells. This was demonstrated by flow cytometry as well as western blots of whole cell lysates and immunoprecipitates yielding a 55kDa band under non-reducing conditions. Anti-FLAG immunoprecipitates probed with an anti-phosphotyrosine mAb yielded a similar major band, and may indicate the recruitment of SHP-1. An additional weaker 110 kDa band was observed in some experiments, suggesting that mouse KIR3DL1 partially exists as a disulfide-linked dimer in 293T cells.

Present studies

4.1. Scope of the thesis

This thesis is divided into two parts. The first and major part describes the cloning and characterization of rodent orthologs to the human KIR family. This discovery led ultimately to the identification of other rodent orthologs to human genes. The specific aims of the work were to:

a) Analyze genomic trace sequences and ESTs available to detect the presence KIR orthologs in the mouse and rat genomes.

b) Identify and characterize the receptors if discovered.

c) Determine phylogenic relationship, chromosomal localization and tissue expression of rodent KIR. Finally, generate expression constructs in order to verify surface expression of the receptor.

As we succeeded in identifying rodent KIR orthologs, we decided to extend our search to include other rodent genes. Genome assemblies had just become available, and this led us to:

e) Generate gene maps of the LRC in mouse and rat based on genomic assemblies and furthermore, resolve similarities and differences with regard to the human LRC.

f) Identify and characterize novel rodents genes, in specific rodent orthologs to human *CD89*.

5.2. The leukocyte receptor gene complex (LRC)

The human LRC on chromosome 19q13.4 contains 45 genes including a selection of 30 genes encoding Ig-like receptors. The centromeric part of the complex is limited by a group of genes unrelated to the Ig-like receptors, the LRC-encoded novel genes (LENGs). The telomeric end of the complex is comprised of a group of structurally related genes including *CD89*, *NKp46* and *GPVI* [39]. The organization of its syntenic regions in mouse and rat is less clear, but some findings are puzzling:

1) KIR receptors in mouse are located on chromosome X, and the Gp49 family (Ig-like receptors which are related to the LILR family in human) is positioned on chromosome 10.
2) In contrast, the LRC in the rat appears to be intact, and shares structural similarity with the human LRC, also regarding Gp49s and KIR.
3) Despite extensive effort, no *CD89* ortholog has been identified in the mouse, and importantly, the presence of a rat ortholog has largely remained uninvestigated.
4) No rodent orthologs of human LAIR have been identified.

5.2.1. Identification of a rat CD89 homolog

Analyzing the mouse genomic assembly, NCBI build 30 (*http://www.ensembl.org/Mus_musculus/*), a retroviral integration site was retrieved at the locus syntenic to the localization of human *CD89*, upstream for *NKp46*. In human, the neighboring KIR locus is positioned further upstream for *CD89*. No *CD89* ortholog was detected in the mouse genomic assembly, however analyzing rat genomic sequences a promising candidate was

detected at the predicted site between *NKp46* and *Kir3dl1*. Based on the human *CD89* cDNA sequence all exons were retrieved, and primers specific for the 5'UTR and 3'UTR regions generated. The gene was successfully amplified from a PVG spleen cDNA library, and subsequent sequencing indicates that the gene does indeed represent a rat *CD89* ortholog. KIR is in rat localized upstream for the *CD89* ortholog and the *Gp49s* are located upstream of the *Kir3dl1*, analogous to the organization of the human LRC provided that *Gp49s* share common parentage with the human LILR family. Together, these findings make it tempting to speculate that the integration of a virus or transposable element in the mouse genome, led to the accidental loss of mouse *CD89* and the translocation of Gp49 and KIR.

Figure 4:

Amino acid sequence of rat *CD89* aligned with human and bovine *CD89* (GenBank accession number X54150 and AY247821, respectively). The alignment was generated by ClustalX, and gaps were introduced to maximize homology. Identical amino acids are represented by dashes. Conserved cysteines in the extracellular domains (involved in the creation of disulfide bonds) are indicated by green asterisks. Blue boxes identify N-linked glycosylation sites (NxS/T). Arginine present in the transmembrane domain, permitting association with the FcεRIγ, is colored red. Predicted amino acid sequence of rat CD89 is 54% identical to human CD89 and 54% identical to bovine CD89.

5.2.2. Cloning of a Leukocyte-Associated Immunoglobulin-Like Receptor (Lair) in the mouse and rat, and a novel family of Ig-like receptors.

Following the approach outlined above, rat and mouse orthologs of human LAIR were identified and successfully cloned, and in addition a novel family of receptors in the mouse and rat LRC detected, denoted LILR.

Figure 5:

Amino acid sequences of mouse *LilrA1*, rat *LilrA1* and rat *LilrA2* aligned with human *ILT11* and human *LILRA1b*. *LILRA1b* appears to be an alternatively spliced variant of *LILRA1a*, but differs in the absence of the third and fourth Ig-like domain of *LILRA1a*. Cysteines are indicated with green asterisks, and blue boxes represent N-linked glycosylation sites. Arginine present in the transmembrane domain is colored red (arginine allows association with the FcεRIγ in the human). Comparisons of the predicted amino acid sequences of *mLilrA1*and *rLilrA1* with *hILT11* and *hLILRA1*, reveal that these genes are closely related. Mouse and rat *LilrA1* are 84% identical to each other, between 56-58% identical to *hILT11* and 54% identical to *hLILRA1b*. The predicted amino acid sequence of *rLilrA2* is 51% identical to *rLilrA1*, 57% identical to *hILT11* and 55% identical to *hLILRA1b*.

Figure 6:

Amino acid sequences of mouse and rat *Lair1* aligned with human *LAIR1* (GenBank accession number AAB69324). Cysteines are indicated with green asterisks, and blue boxes represent N-linked glycosylation sites. Intracellular ITIM-motifs (potentially involved in signal transduction by binding SHP-1) are colored red. *mLair1* and *rLair1* are 72% identical to each other, and 48% and 46% identical to *hLAIR1* respectively.

The predicted overall structure of these receptors conforms to a type I transmembrane protein comprised of two extracellular Ig-domains, a transmembrane domain containing the charged amino acid arginine, suggesting interaction with adaptor proteins such as FcεRIγ and DAP12, and a short cytoplasmic region devoid of any signaling motifs. These receptors do not represent a "missing link" between the human LILR family and the rodent *Gp49* and *Pir* families. However, as depicted in the dendrogram, human LILR and rodent *Gp49*, *Lilr* and *Pir* do constitute a distinct family, and obviously share common ancestry. Probably, strain to match the fast diversification of pathogens led to expansion of the family, and in rodents the family over time separated into three largely related subclasses.

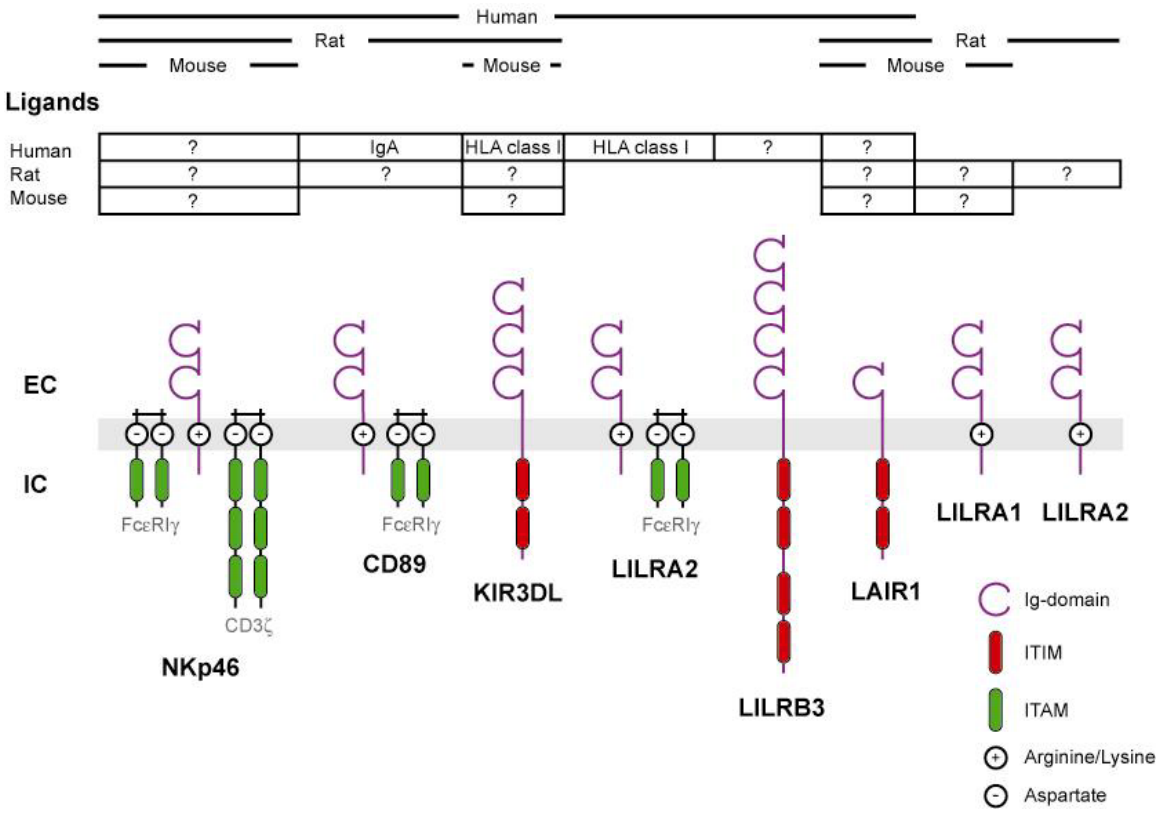


Figure 7:

Schematic representation of human LILRB3 and novel rodent LILR genes, along with CD89, LAIR, NKp46 and KIR. The horizontal lines at the top indicate the species in which known homologs have been identified. Ligands for the various receptors are shown in the boxes at the top of each receptor. EC, extracellular domains. IC, intracellular domains. The transparent grey line represents the transmembrane region. Horizontal interchain lines indicate disulphide bridges.

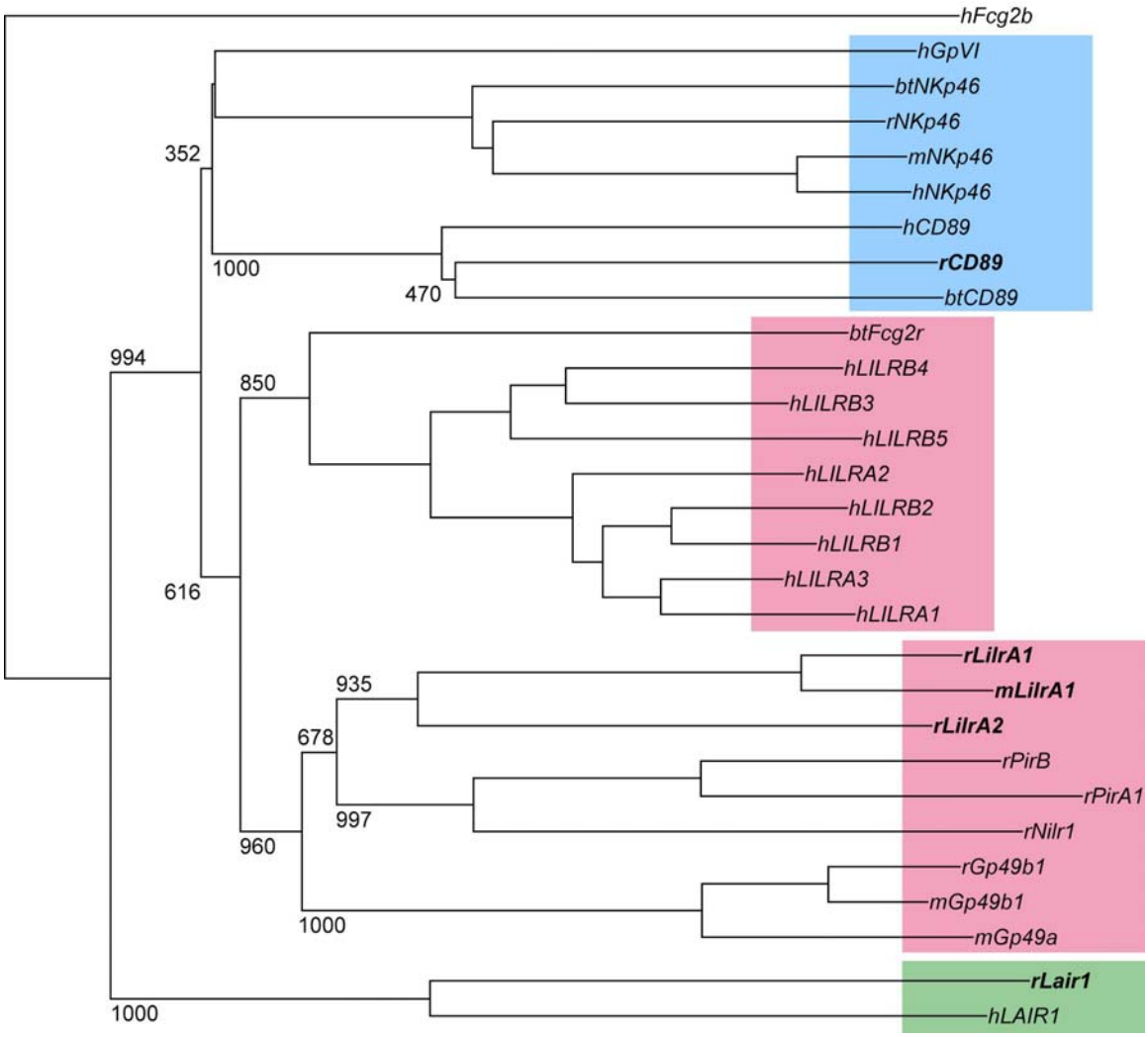


Figure 8:

Phylogram displaying similarity between the novel family of rodent LILR receptors, rat *CD89*, rat *Lair* and selected IgSF leukocyte receptors in the LRC. Amino acid sequences of each receptor were aligned using ClustalX, and tree topology was estimated by neighbor-joining where branch values represent the number of bootstrap support after 1000 replications. Colors highlight closely related genes. *hFcg2b* is defined as an outgroup. h, *Homo sapiens*; m, *Mus musculus*; r, *Rattus norvegicus*; bt, *Bos taurus (cattle)*.

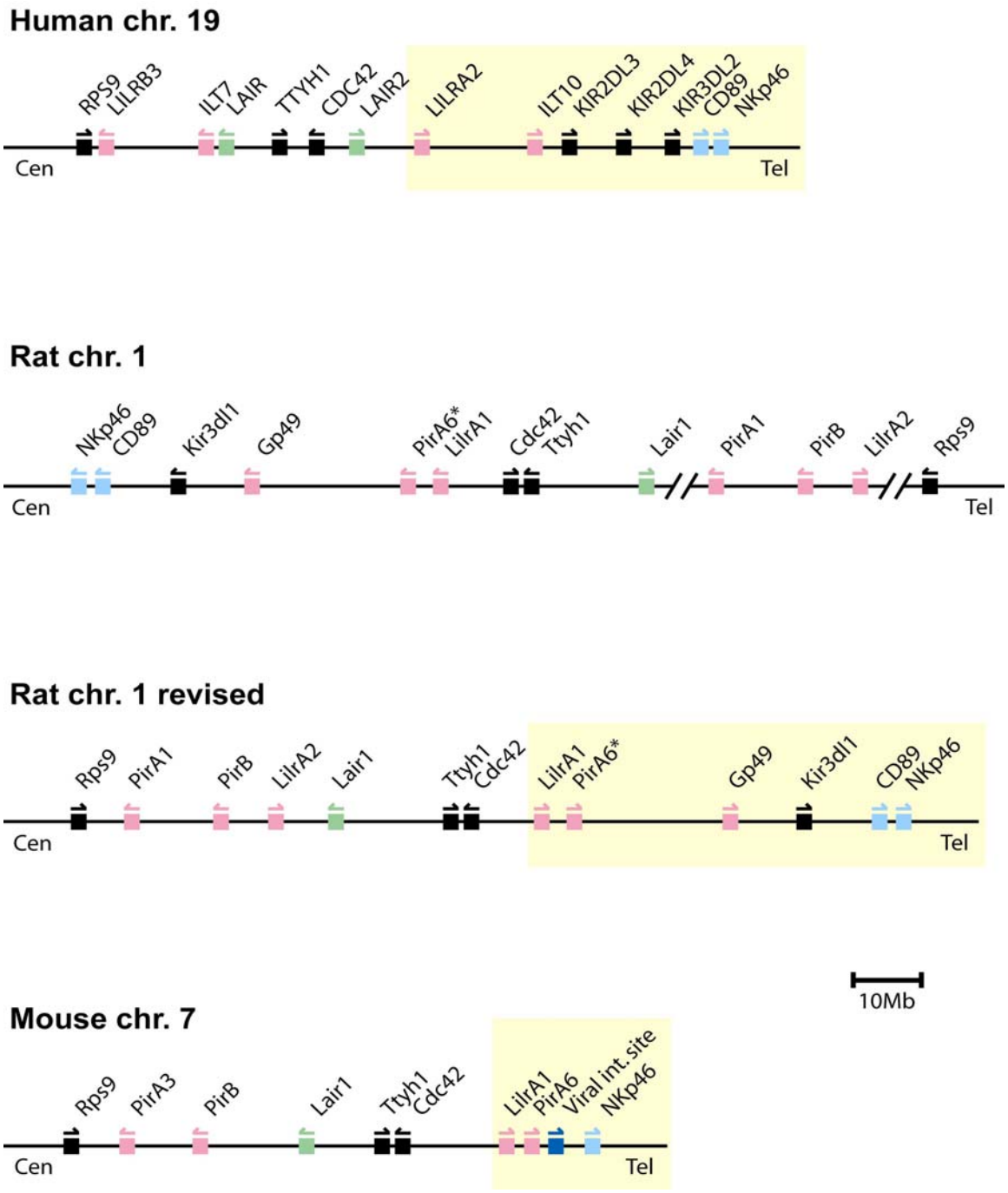


Figure 9:

Genomic organization of the LRC in human, mouse and rat, based on available genome assemblies (*http://www.ensembl.org*; *http://www.ncbi.nlm.nih.gov*). The human19q13.4 region is depicted along with its syntenic regions on mouse chromosome 7 and rat chromosome 1. The ribosomal protein RPS9 represents the centromeric border of the LRC, and *NKp46* limits the locus at the telomeric end. Several genes encoding LILR receptors, PIRs, KIRs as well as several non-IgSF genes have been omitted for clarity. Diagonal lines represent possible errors in the rat assembly, the present assembly is also in conflict with our obtained RH data. A reconstructed assembly consistent with our data has been included. Human sequence containing *NKp46*, *CD89*, KIR and the telomeric LILR cluster are together with equivalent regions in the mouse and rat genomes, demarcated with transparent yellow boxes. The viral integration site present in mouse, possibly representing the site of translocation is denoted with a dark blue box. **PirA6* has not been characterized in the rat. *PirA6* as depicted in the rat LRC represents genomic sequence sharing amino acid similarity to mouse *PirA6*, suggesting the presence of a rat *PirA6* homolog.

V. Discussion

HLA matched allogeneic hematopoietic transplantations have revolutionized the treatment of leukemia and lymphoma. T-cells contained in the transplant are vital in eradicating malignant cells (graft verses leukemia effect GVL) and in reconstituting immunity, but unfortunately the T-cells attack other tissues in the recipient as well (graft verses host disease GVHD). Recently, knowledge of the KIR receptors have led to radically improved outcome in the treatment of acute myeolid leukemia (AML). It is believed that the KIR receptors expressed by an NK cell precursor are determined by random processes, and that NK cells expressing receptors for self HLA class I molecules are selected. Thus, NK cells from any given person will be alloreactive toward cells from other individuals who lack their KIR ligands, and conversely, tolerant of cells from an individual who shares the same KIR ligands. Clinical transplantations in a group of high risk leukemia patiens lacking a HLA matced donor have shown that mismatching the three major KIR ligands; HLA-C group 1,2 and HLA-Bw4, protect patients from rejection, AML relapse and unexpectedly reduce the severity of GVHD [65]. The reason why alloreactivity does not cause GVHD but actually protects against it is believed to be alloreactive NK cells killing recipient antigen presenting cells [65]. Alloreactive NK cell infusions have the potential to improve outcome even further and are of great therapeutic interest.

By cloning the first KIR receptors in rodents better animal models may be introduced in the ongoing investigations of the KIR receptors, but first we need to clarify if the receptors play a similar role in rodents as they do in human. Some of the techniques summarized below will be of interest regarding other novel receptors we have identified, and will hopefully divulge some important results.

The mouse KIR3DL1 receptor contains two ITIM motifs in the cytoplasmic domain indicating an inhibitory function. The next step is to elucidate the receptors signaling mechanisms and to resolve the function of the protein. A chimeric construct comprising the CH2 and CH3 domains of mouse IgG2b heavy chain bound to the extracellular

domains of KIR3DL have been made. Soluble fusion protein can be generated by transfecting 293T cells with the construct, followed by protein purification on a protein G column. Finally, rats will be immunized with the fusion protein over a three month period. We have previously succeeded in generating several antibodies at our laboratory using this method. An antibody gives us the opportunity to carry out immunoprecipitations and facs flow analysis on cells isolated from mice, and will be an important tool in dissecting the proximal signaling events as well as performing expression studies.

RNK_{DA1} is a rat NK cell line isolated from DA rats. This cell line provides a better model for in vitro studies than 293T cells. Stable transfection of these cells with KIR3DL1 can be achieved by electroporation with a linearized plasmid containing the gene and a selection marker. Cytotoxicity assays will complement the studies of signaling mechanisms, and give us important information about the receptors effector functions.

The identification of the ligands is of pivotal importance, but may prove difficult to accomplish. The KIR-Fcγ2b fusion protein can be used as a probe in facs flow analysis binding transfected target cells, but the method may turn out elaborate and time consuming even though MHC class I molecules are suspected ligands. Another attractive approach is to utilize a green fluorescent protein (GFP)-reporter cell line made by Arase and Saito, these cells are stably transfected with a GFP controlled by NF-AT. The cell line is transfected with a chimeric construct comprising the extracellular domains of KIR3DL1 bound to the transmembrane and cytoplasmic parts of the TCR ζ chain. If these cells are incubated with target cells expressing a ligand for KIR3DL1, NF-AT is activated by the TCR ζ part of the chimeric protein, and the cells turn green following a few hours.

VI. Acknowledgements:

This thesis is the result of my work carried out in the period 2002-2004 at the Department of Anatomy, Institute of Basic Medical Sciences, University of Oslo. Throughout this period I have received a partime student fellowship from the Norwegian Cancer Society. My work has also received financial support from the Norwegian Reasearch Council and Odd Fellow medisinsk-vitenskapelig forskningsfond.

I am grateful for the help and inspiration I've received from my friends, past and present colleagues, who all have contributed in making this period inspiring and long lab hours worthwhile. In specific I would like to thank:

My supervisor Erik Dissen for excellent guidance and the enormous freedom I've received in choosing my own projects and pursuing my own goals and ideas. Always supportive and always encouraging.

My co-supervisor Sigbjørn Fossum , his broad knowledge of immunology is invaluable and together with Erik he provides a unique and stimulating environment.

Øivind Nylenna, Per Christian Sæther, Ingunn H. Westgaard and Line Flornes for lots of help and a very large number of hours spent together in the lab.

Marit Inngjerdingen, Lise Kveberg, Megan Wittman, Shirin Nejad, Stine Granum, Vibeke Sundvold Gjerstad, Thorny Andersen, Ke-Zheng Dai, John Torgils Vaage, and Christian Naper for providing a great atmosphere.

Marianne Kollerud and Wendi Jensen for technical assistance.

My family for patience and support, and last, but never least Na for always being there.

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